

# Scrapie



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Minireview

# Scrapie—Uncertainties, biology and molecular approaches

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**Abstract**

The study of the biology of scrapie in sheep is irretrievably associated with the genetics of the PrP gene in sheep. Control of susceptibility and resistance is so closely linked to certain alleles of the sheep PrP gene that no review on scrapie can avoid PrP genetics. Before the importance of PrP protein was discovered and before the influence of the gene itself on disease incidence was understood, it was clear there were some sheep which were more susceptible to natural scrapie than others and that this feature was heritable. These early observations have led to the development and use of PrP genotyping in sheep in what is probably the biggest genetic selection process ever attempted. The accompanying increase in surveillance has also discovered a novel type of scrapie, the subject of much speculation about its origin.


*Keywords:* Natural scrapie; Atypical scrapie; Sheep; PrP genetics; Susceptibility; Resistance

## 1. Scrapie susceptibility studies before the discovery of the PrP gene

Studies of natural scrapie in sheep field conditions do not have the luxury of pathogenesis experiments in laboratory rodents where every aspect is controlled, particularly the time of initial infection. However, from the earliest reports of the disease in sheep, it was noticed that family lines had a strong influence on the occurrence of scrapie and the measures put in place to control the disease relied heavily on this fact. In the history of sheep breeds and scrapie written by Parry [1] and published after his death, details of these attempts at disease control are described. In the 18th and early 19th centuries in Britain, scrapie was a severe problem in certain breeds of sheep such as Dorset Horns and Hampshires in the county of Wessex. It was also noticed that using outbred rams could reduce or control the disease. Parry and others believed that scrapie was a simple genetic disease because the familial link was so strong; however this is now considered unlikely. Both a susceptible genotype and an infection are required before disease develops [2,3]. When in more recent times scientists began to study natural scrapie in sheep, the familiar influence on its occurrence was put to the test.

Early experimental studies of transmission of scrapie using sheep often gave confusing and contradictory results. One of the first attempts at a research programme (in England in 1952) used Welsh Mountain sheep because of the breed's small size and low cost. However, after three experiments, only 5 of 156 inoculated sheep developed scrapie, an infection rate of 3%. A group of South Country Cheviots was a little better giving 14% and 35% affected sheep in two separate experiments; however, these rates would render experiments on scrapie in sheep prohibitively expensive [4]. It was thought that some breeds would be naturally more susceptible than others and so a large study of British sheep was set up to find a suitable breed to use for pathogenesis studies [5]. Twenty-four different breeds of sheep, over 40 animals per breed on average and a total of 1,027 animals were injected intracerebrally (ic) with SSBP/1 scrapie [6] (Table 1). There was a range of levels of susceptibility running from over 70% in Herdwicks and Dalesbreds to zero (apparently complete resistance) in Dorset Downs. Swaledales were 54% susceptible but all other breeds gave much lower disease rates with, for example, Scottish Blackface at 8% and Welsh Mountains at 4%. Incubation periods ranged from 3.5 months to 23 months and the experiment was brought to an end in July 1959, two years (24 months) after the scrapie injections were carried out. Later studies gave different results with Herdwicks at 30% susceptible [7] and in the original group

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Table 1
Breed study of scrapie susceptibility initiated in 1957

| Breed | Number inoculated % | Affected with scrapie |
|---|---|---|
| Herdwick | 36 | 78 |
| Dalesbred | 43 | 72 |
| Swaledale | 46 | 54 |
| South Country Cheviot | 45 | 36 |
| Derby Gritstone | 46 | 35 |
| Exmoor Horn | 41 | 34 |
| Border Leicester | 42 | 26 |
| Scottish Blackface | 44 | 18 |
| South Devon | 35 | 17 |
| Romney Marsh | 43 | 16 |
| Welsh Cheviot | 40 | 15 |
| Ryeland | 34 | 15 |
| Dorset Horn | 45 | 13 |
| Suffolk | 51 | 12 |
| Leicester | 42 | 12 |
| Welsh Mountain | 42 | 10 |
| Hampshire Down | 30 | 10 |
| North Country Cheviot | 45 | 9 |
| Southdown | 38 | 8 |
| Wiltshire Horn | 57 | 7 |
| Shropshire | 41 | 5 |
| Kerry Hill | 41 | 2 |
| Clun Forest | 52 | 2 |
| Dorset Down | 48 | 0 |

Sheep inoculated with SSBP/1 scrapie, 10% brain homogenate.
Taken from reference [5].

of "resistant" Dorset Downs which were left alive after the experiment was supposedly finished, some did actually go on to develop scrapie at later dates [6]. It became obvious that there was as much variation between rates of susceptibility between individual animals as there had been thought to have been between breeds and it became clear that flocks of sheep of predictable susceptibility and resistance were needed for useful pathogenesis studies.

## 2. Development of sheep lines selected for resistance and susceptibility to scrapie

Three lines of sheep in the UK were produced using challenge with scrapie as the means of selection. Breeding lines were set up using animals proven to be susceptible and/or resistant to this challenge (Cheviots, Herdwicks and Swaledales) [4,8,9]. It is possible now to do this using PrP genotype selection very much more quickly but the immense amount of work which went into producing these flocks is in danger of being forgotten and is important as the selection lines allowed us to develop the PrP genetics with solid biological information to underpin the work. The Herdwick flock is now reduced to minimal numbers and is held on the NPU farm (see below). The Swaledale flock has been very recently returned to normal commercial farming and is effectively lost for scientific study (Merrell, personal communication).

The Cheviot sheep flock (held on the farm of the Neuropathogenesis Unit (NPU) Edinburgh) was started in 1961 from a foundation flock of 303 ewes and 15 rams with no record of exposure to scrapie. They were split into two lines (positive and negative) based on their responses to injection with SSBP/1 scrapie [6,8]. All foundation animals were injected (usually subcutaneously, sc) with SSBP/1 after mating (males) and after lamb weaning (females). The policy of challenging every animal was continued for many years to consolidate the selection lines and to study the genetics of disease control. Animals were mated up to three times prior to challenge but were never mated after scrapie challenge and careful records were maintained to avoid inbreeding as much as possible. Animals developing scrapie by around 500 days post infection formed the basis of the positive line and were assigned to the negative line if experimental scrapie did not develop within 2 years of sc challenge [10].

From line cross and back cross segregation experiments it became clear that a single gene (given the name *Sip*, standing for scrapie incubation time) controlled susceptibility to SSBP/1. *Sip* had two alleles, *sA* and *pA* [11]. The negative line were all *Sip pApA* and the positive line were mostly *Sip sAsA* or *sApA,* albeit with varying low numbers of *Sip pApA* in the positive line as a result of heterozygote matings. The continued challenge with SSBP/1 injection revealed these resistant sheep but this was a very long drawn out process. The flock, now known as the NPU Cheviot flock, is still in existence and being used for pathogenesis, PrP genetics and epidemiology studies but we now understand that the *Sip* gene encodes the PrP protein and the older terminology is no longer in use. It does help however in the understanding of the older papers to realise that the *Sip sA* allele is equivalent to the sheep PrP VRQ allele (see below). The *Sip pA* allele group includes every other sheep PrP allele, for example ARQ, ARR, AHQ.

## 3. PrP gene and genetics

In mice the PrP gene was known to have polymorphic variants which could be linked with control of scrapie incubation time [12]. Mice have been used for many decades to study sheep scrapie strains as scrapie from many field cases will transmit to mice. Mouse passaged strains with varying incubation period and pathology have been immensely useful in providing information about which cells are important in scrapie transmission and in studies of susceptibility and resistance [13]. The subsequent development of transgenic lines of mice expressing PrP genes from other species or with specific mutations has in more recent years transformed the way we understand these diseases. Rodent models are outwith the scope of this review but there are many other sources of information which can be consulted on the subject ( for example [14,15]). Because the mouse PrP gene showed amino acid variation linked to disease control, it seemed an obvious step to examine DNA from the selection line of the NPU Cheviots for a similar marker. The initial studies were carried out using restriction enzymes and generating RFLPs (restriction fragment length polymorphisms). Two of these showed variation which was associated with the susceptible or resistant line, *Eco*RI and *Hin*dIII. Nine other enzymes were also tested and failed to show any variation.

Table 2
Association of PrP gene *Eco*RI restriction site polymorphism with scrapie susceptibility in NPU Cheviot sheep

| Fragments hybridising to PrPcDNA | Sip genotype predicted from breeding records (PrP genotype subsequently assigned) |
|---|---|
| *Eco*RI | |
| 6.8 kb DNA fragment only | sAsA (PrP VRQ/VRQ) |
| 4.4 kb DNA fragment only | pApA (PrP AXX/AXX *) |
| Both fragments | sApA (PrP VRQ/AXX) |

Data adapted from reference [13].
* X=any of the possible amino acids at these codon positions.

In a study of 32 sheep, 10 negative line and 22 positive line, these RFLPs were reliable in identifying which sheep belonged to which line [16] (Table 2); however, when the study was extended to natural scrapie samples from throughout the UK, only the *Eco*RI RFLP remained reliable [17]. From subsequent [18] studies we have demonstrated that the polymorphism which alters the *Eco*RI recognition sites lies within the 3′ untranslated region of the sheep PrP gene and does not affect the protein sequence (Fig. 1). Despite this, the RFLP remains a reasonably good marker for susceptibility in a wide range of breeds of sheep (Goldmann, personal communication).

For predicting which animals would succumb to natural scrapie infection in the normal farm situation, the RFLPs were not 100% accurate and as it was thought likely that a polymorphism which affected the amino acid sequence of the gene would have more profound effects on pathogenesis, a DNA sequencing search of thousands of sheep PrP gene alleles started and continues to this day, new polymorphic codons being described regularly. The first codon known to be polymorphic was codon 171 [19] where either arginine (R) or glutamine (Q) could occur. Subsequently two other codons were found to be important: 136 with valine (V) or alanine (A) and 154 with histidine (H) or arginine (R) [20] (Fig. 1). Many other codons are now known to be polymorphic but these three codons remain of major importance. The first studies of incidence of these polymorphisms in scrapie sheep looked at each codon individually and in turn. Experimental challenge studies in NPU Cheviots suggested that with SSBP/1, V at codon 136 ($V_{136}$) on at least one allele was needed in order to transmit scrapie, whereas $A_{136}$ was associated with resistance [20]. This was supported in a study of natural scrapie in some breeds in France [21] but the $V_{136}$ allele clearly did not occur in some breeds [22] including Suffolks and in those cases scrapie occurred in animals homozygous for Q at codon 171 ($QQ_{171}$). The clear association of $QQ_{171}$ with natural scrapie in Suffolk sheep was first shown by Westaway [23] in a study which indicated that 100% of 31 unrelated scrapie-affected Suffolk sheep in the USA were of $QQ_{171}$ genotype whereas in the healthy controls ($n$=69) genotype frequencies were 51% $QQ_{171}$, 43% $QR_{171}$ and 6%$RR_{171}$. The result is highly significant and it remains true that $QQ_{171}$ animals are more likely to develop scrapie than the other genotypes; many animals of $QQ_{171}$ genotypes remain healthy and live long lives. It is clear that it is a subgroup of $QQ_{171}$ sheep which is most susceptible and that this subgroup has V at codon 136—but only in breeds which encode $V_{136}$ [24]. In breeds which do not encode $V_{136}$ (including Suffolks) it is not known what distinguishes susceptible $QQ_{171}$ animals from resistant $QQ_{171}$ animals. To explain further it is helpful to use the now conventional terminology to describe sheep PrP genotypes using the 3 codon format which gives each codon in turn for each allele in turn. In breeds such as Cheviots, Swaledales and Shetlands, it is the presence of V at codon 136 (the VRQ allele) which is linked to scrapie [18,25,26]. Susceptible sheep are likely to be VRQ/VRQ or VRQ/ARQ genotypes. These are still QQ at codon 171 of course, but ARQ/ARQ animals in these flocks will survive. In some breeds such as Suffolks, the VRQ allele is so rare as to be non-existent and the most susceptible sheep are ARQ/ARQ genotype as described previously [23,27] however as stated above, not all animals of this genotype group will develop disease and it is not known what differentiates susceptible ARQ/ARQ sheep from resistant ARQ/ARQ sheep. The ARQ allele is not a single entity but can be divided up into subgroups based on polymorphisms at other codons. None of these; including also polymorphisms in the DNA in the promoter region, has so far shown universal links with disease (Goldmann, personal communication).

## 4. The genetic disease hypothesis

In flocks in the UK which have sheep encoding the VRQ allele, any sheep with a homozygote genotype (VRQ/VRQ) is in some outbreaks virtually guaranteed to develop natural scrapie. In the NPU Cheviot flock which has endemic natural scrapie, 100% of VRQ/VRQ animals succumb to natural scrapie at about 2 years of age [10]. The link is so strong that it could be interpreted as being a genetic disease, without any infection being involved. This idea is an old one, suggested by the very obvious familial occurrence of scrapie in sheep lines [1]. However, it has now been refuted. Not only are VRQ/VRQ sheep found in countries such as Australia and New Zealand, which do not have endemic natural scrapie [2,3], but it has recently been shown that VRQ/VRQ sheep from the NPU Cheviot flock can be reared to a healthy old age free of scrapie if they are born in clean surroundings and maintained there [28]. For sheep without the VRQ allele, such as Suffolks, it is also true that the susceptible genotype ARQ/ARQ is common in sheep in scrapie free

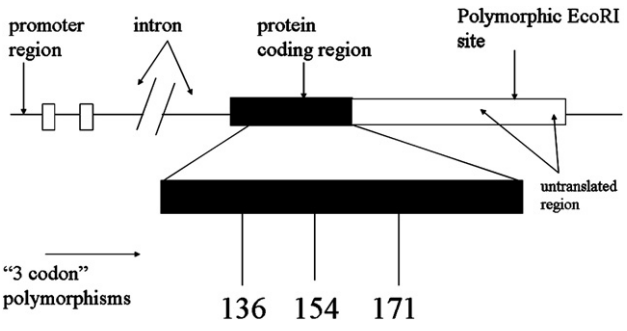


Fig. 1. Diagram of sheep PrP gene structure showing the positions of polymorphisms discussed in the text.

Table 3
Association of PrP gene coding region polymorphisms with scrapie in NPU Cheviot sheep following sub-cutaneous injection with SSBP/1

| PrP genotype | Incubation period in days (SD) | Survival, no scrapie (days) |
|---|---|---|
| VRQ/VRQ | 180 (20) | – |
| VRQ/ARQ | 269 (76) | – |
| VRQ/ARR | 352 (63) | – |
| ARQ/ARQ | – | 998–2330 |
| ARQ/ARR | – | 820–4250 |
| ARR/ARR | – | 1150–2720 |

Data adapted from reference [29].

countries [2]. Perhaps, as some suggested, sheep from New Zealand are simply not susceptible to scrapie, even with the genotypes (e.g. VRQ/VRQ and ARQ/ARQ) which develop scrapie in the UK. By direct challenge, infecting sheep experimentally with SSBP/1 scrapie, it was first shown that Cheviot and Poll Dorset sheep from New Zealand and encoding the VRQ allele were indeed susceptible to scrapie. Incubation periods were however slightly different from those in UK sheep, for example VRQ/ARQ and VRQ/ARR sheep having similar incubation periods rather than the 100 days difference seen with NPU Cheviots [29] (compare Tables 3 and 4). In the UK, DEFRA has built up a flock of sheep originating in New Zealand and therefore free of endemic scrapie. These animals have been used in many studies from many laboratories and are indeed fully able to develop both scrapie and BSE from inoculationed infected material.

It is now absolutely clear that in order to develop scrapie, an animal has to have both a susceptible PrP genotype AND be exposed to an infecting agent.

## 5. Use of PrP genotype information in sheep breeding

Although the sheep PrP gene is complex in terms of how many codons are polymorphic, the genotype which is usually used is the three codon genotype (136 154 and 171). Using these codons, there are five commonly occurring alleles: VRQ, ARQ, ARR, AHQ and ARH [30]. Many studies have been carried out in sheep from different countries which confirm this information is not limited to UK sheep.

In the USA, the great majority of reported scrapie cases are in sheep of Suffolk origin and, like all Suffolks, these tend to have only three genotypes ARQ/ARQ, ARQ/ARR and ARR/ARR [23]. This makes breeding against scrapie susceptibility very easy. ARQ/ARQ is the susceptible genotype and in the US, no cases have been found in ARQ/ARR [31] although some sheep with this genotype do develop scrapie in Suffolk sheep in Europe [27]. There are however in the USA, some breeds of sheep encoding the VRQ allele [32] and these would be expected to be scrapie susceptible if exposed to infection. Within Europe there are breeds with genetics similar to Suffolks but the sheep breeds frequently have more complicated genetics; for example in breeds such as Texels, there are 15 different genotypes [33] and as a result selection is more difficult and takes longer.

In the UK in 2001 the National Scrapie Plan (NSP) was put in place to assist in sheep breeding to reduce the frequencies of the susceptible genotypes. This was partly due to worries that BSE may have infected the national flock as, if it had done, it would represent a danger to the human foodchain. Within the NSP, there are 15 genotypes listed in 5 different groups relating to the known susceptibility to natural scrapie; for example NSP type 1 is ARR/ARR, which has high resistance and NSP type 5 contains VRQ/VRQ, ARQ/VRQ, ARH/VRQ, AHQ/VRQ which have high susceptibility themselves and will also pass on the high risk allele VRQ to their offspring (Table 5). The aim of the NSP is to reduce the number of sheep with the most susceptible genotype to scrapie (VRQ/VRQ) and to increase the number of sheep with the more resistant genotypes. The scheme has run through various programmes including the Compulsory Ram Genotype Scheme (CRGS) which was designed according to European legislation which required all member states to establish such genotyping and breeding plans for TSE resistance. The CRGS aims to remove rams with the most susceptible genotypes and is a legal requirement for all sheep farmers who own purebred breeding flocks. With this and other schemes in progress, the NSP has tested over 2 million sheep to date [34]. With the similar programmes taking place within the

Table 4
Association of PrP gene coding region polymorphisms with scrapie in New Zealand-derived Cheviot sheep following sub-cutaneous injection with SSBP/1

| PrP genotype | Incubation period in days (SD) | Survival, no scrapie (days) |
|---|---|---|
| VRQ/VRQ | 150 (11) | – |
| VRQ/ARQ | 256 (39) | – |
| VRQ/ARR | 259 (9) | – |
| ARQ/ARQ | – | >1000 [a] |
| ARQ/ARR | – | >1000 |
| ARR/ARR | – | >1000 |

Data adapted from reference [29].
[a] Clinical cases have occurred in this genotype of these sheep at over 1000 days post inoculation but still have to be confirmed by pathology and biochemistry.

Table 5
National Scrapie Plan, Britain

| PrP genotype | NSP [a] type | Degree of resistance |
|---|---|---|
| ARR/ARR<br>ARR/AHQ<br>ARR/ARH<br>ARR/ARQ | 1 | Sheep that are most resistant to scrapie<br>Sheep genetically resistant to scrapie but need careful selection when used for breeding |
| ARQ/ARQ<br>AHQ/ARH<br>AHQ/ARQ<br>ARH/ARH<br>ARH/ARQ<br>ARQ/ARQ | 3 | Sheep genetically have little resistance to scrapie and need careful selection when used for breeding |
| ARR/VRQ | 4 | Sheep genetically susceptible to scrapie and should not be used for breeding unless in the context of a controlled and approved breeding programme |
| VRQ/AHQ<br>VRQ/ARH<br>ARQ/ARQ<br>VRQ/VRQ | 5 | Sheep that are highly susceptible to scrapie and should not be used for breeding |

Table adapted from [86] (Crown copyright).
[a] NSP—National Scrapie Plan.

European Union countries, this has to be the largest genetic selection programme ever put in place and is generating a huge amount of data on genotype frequencies in different sheep breeds from each country and region including France [24], Spain [35], Portugal [36], Greece [37], Italy [38], Slovakia [39] and Germany [40]. There is also similar information from Iceland [41] where there is the added difficulty of the apparent complete lack of ARR/ARR sheep for resistance breeding—something which is also a problem for breeds in Britain known as "rare breeds" in which total numbers are small [42].

In conjunction with the genotype information, there is also EU legislation relating to fallen stock and to sheep and goats at abattoirs which advocates searching for any sign of TSE infection using EU approved rapid testing methods which include those of Biorad TeSeE, Prionics, Enfer, and InPro [43]. All this testing of many thousands of animals has resulted in the discovery not only of the expected pre-clinical scrapie cases, but also an apparently different form of scrapie known as atypical scrapie which will be discussed below in detail. The original familiar scrapie is now known as classical scrapie.

## 6. BSE in small ruminants

Part of the reason for the great interest in TSEs in small ruminants and the enormous amount of work and tax payers' money involved, is because of bovine spongiform encephalopathy (BSE) which, as a disease of cattle, entered the human foodchain and resulted in the devastating disease of vCJD [44,45]. The most widely accepted theory on the origin of BSE is that infection was recycled by feeding ruminant derived meat and bone meal protein supplements to cattle [46]. It is thought likely that sheep and goats were also exposed to the source of BSE infection in their food and it is known that small ruminants are susceptible to BSE infection when experimentally challenged, either by injection or by the oral route using only 0.5 g bovine brain [47,48]. The use of animals whose PrP genotype was known, allowed the genetics of BSE susceptibility in sheep to be determined—the genotype ARQ/ARQ has the shortest incubation periods for example [49,50]. However in several studies it has been found that not every sheep of ARQ/ARQ genotype is actually susceptible to BSE infection [51]. Recently more detailed analysis has suggested that in experimental challenges with BSE, sheep of genotypes encoding the ARQ allele but also with leucine (L) at codon 168 (ARLQ) have increased resistance to infection; in other words genetic linkage with TSE disease is not always straightforward [52,53].

ARQ/ARQ is a genotype which is also linked to susceptibility to Classical Scrapie [30] and to experimental challenge with CH1641 scrapie [49]. However BSE exhibits characteristics which allow (in controlled conditions) its discrimination from scrapie. The BSE phenotype includes the patterns of incubation periods when injected into a specific panel of mouse strains [44,54], the Western blot signal from $PrP^{SC}$ [45] and immunohistochemistry (IHC) detection of $PrP^{d}$ patterns in the brain and using antibodies with different epitopes on the PrP molecule [55,56]. ($PrP^{d}$ is a term used when the disease associated form of PrP protein is detected using methods without a protease digestion step.) Mouse transmissions are both costly and time consuming and for most research labs the Western blot pattern is the first choice, although if BSE is suspected, a battery of other tests is required for confirmation [57].

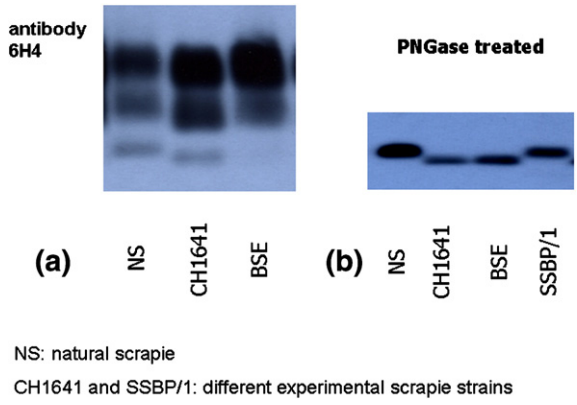


Fig. 2. Western blots of experimental BSE and scrapie in sheep using the antibody 6H4. (a) $PrP^{SC}$ prepared from natural (classical) scrapie (NS), CH1641 scrapie and BSE using proteinase K. (b) PNGase treatment to remove the carbohydrates from PrPSC molecules thus showing a single unglysylated band in natural (classical) scrapie (NS), CH1641 scrapie, BSE and SSBP/1 scrapie.

For Western blotting, $PrP^{SC}$ is extracted using protease K and visualised by polyacrylamide gel electrophoresis and autoradiography producing a pattern of three bands or glycoforms which correspond to diglycosylated, monoglycosylated and unglycosylated forms of the protein [58]. Differences in the apparent molecular weight of the unglycosylated band can give a preliminary identification of BSE as in this case the band runs to a position slightly lower than with most forms of scrapie [59]. BSE also tends to have higher amounts of the diglycosylated band than $PrP^{SC}$ from scrapie brains. This is illustrated in Fig. 2. In panel 2(a) natural scrapie (NS) is compared with BSE and with a scrapie source CH1641. In these preparations the lowest molecular weight (unglycosylated) band is not visible in the BSE cases and so an enzyme PNGase (Peptide:N-glycosidase) can be used to cleave the carbohydrates from the protein and leave a single clear unglycosylated band on the gel (Fig. 2b). The fact that CH1641 scrapie looks similar to BSE adds to the caution with which this technique should be used when attempting to diagnose BSE but CH1641 is quite different from BSE in biology and in patterns of PrP deposition in clinically affected brain [60]. An additional method of BSE detection by Western blot is related to differential binding to antibodies (Fig. 3). BSE $PrP^{SC}$ is detectable using the antibody 6H4 but reacts much less positively with P4 (compare lane 1 in Fig. 3a with lane 1 in Fig. 3b) which has an N-terminal epitope probably cleaved by proteinase K in the BSE samples [59]. Using such techniques backed up with mouse transmissions, BSE was identified in a French goat sample [61] although no further cases have been detected despite increased surveillance. In a very small retrospective IHC study of goats in the UK, four animals which had naturally occurring TSE were compared with experimentally challenged goats which had either scrapie or BSE. Of the four natural TSE cases, two resembled natural scrapie in that brain sections stained with all antibodies tested,

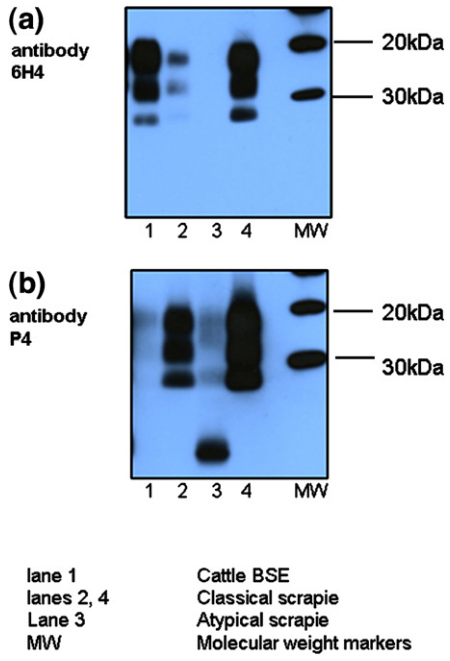


Fig. 3. Western blots of $PrP^{SC}$ from BSE in cattle compared with classical and atypical scrapie in sheep. (a) Antibody 6H4 (b) the same blot stripped and analysed again using the antibody P4.

N-terminal and C-terminal epitopes. One goat however presented antibody intra-neuronal staining patterns identical to those of experimental BSE from goats and sheep in that the N-terminal antibodies did not stain the sections. The fourth goat was intermediate [62]. Transmissions to mice have been set up to further test the BSE-like goat but the extent of variation in just four goat samples is remarkable, when compared with sheep. It is possible that natural TSE strains in goats are more variable than in sheep and it also raises the intriguing and highly speculative possibility that the origin of BSE may have not have been in sheep after all but in goats, although there is no evidence for this.

There have been various attempts to find signs of BSE occurring naturally in the British sheep population. Mathematical modelling and epidemiological studies have looked at various possible scenarios and cannot rule out the chances of finding BSE, albeit at low levels [63]. An estimate of the maximum frequency of cases of BSE mis-identified as scrapie was 5% in one study [64]. This means of course that BSE could be present at any level below 5%, including zero. However a more recent estimate is based on detection of $PrP^{d}$ by IHC and of $PrP^{SC}$ by Western blot in a total of 2,368 scrapie sheep samples from 1998 to 2004 in Britain. The sheep came from 450 flocks and over half of them came from just 34 flocks but, although some samples gave unusual patterns, none were exactly the same as BSE. With a 95% confidence limit this gives a new estimate for BSE to be hidden amongst scrapie sheep at a maximum rate of 0.66% [65].

## 7. Atypical scrapie

The first cases of what are now known as atypical scrapie were discovered in Norway in 1998 and were sufficiently distinct from classical scrapie to be given the name Nor98 [66]. Unlike the pathology found with classical scrapie, there was little or no vacuolation in the obex of the brain, nor was there any accumulated $PrP^{SC}$ in immunohistochemistry of the obex. Instead there was most sheep exhibited vacuolation and $PrP^{SC}$ deposition in the cerebellar region. An additional feature was the Western blot pattern of $PrP^{Sc}$ which exhibited a very small molecular weight band at about 12 kDa, not seen with classical scrapie. When mouse transmissions were attempted, the disease did not transmit to normal mice but did transmit to transgenic mice expressing the VRQ allele of the sheep PrP gene [67]. The genetics of sheep with Nor 98 were also interestingly different from classical scrapie in that Nor98 tends to occur in genotypes more usually associated with some resistance to classical scrapie, particularly with the AHQ allele. In addition an association with another codon outside the "3-codon" genotype, that of codon 141 was found, with phenylalanine (F) being more commonly found than leucine (L) so that Nor98 sheep were more likely to encode ARFQ than ARLQ [68].

Since the discovery of Nor98, more unusual cases have come to light, picked up during the rapid testing being applied for $PrP^{SC}$ detection in sheep and goats throughout Europe. Such large numbers go through this precautionary testing that various commercial kits have been approved after evaluation by the European Food Safety Authority. The kits vary in which method is used to extract protein from brain samples and it is not clear what exactly is in each kit due to commercial confidentiality; however it is clear that for example the method used for the BioRad TeSeE extraction uses a lower level of proteinase K (low stringency) than does for example the Prionics Check kit (high stringency). Labs using the BioRad method started to find positive results from groups of sheep of PrP genotypes regarded as scrapie resistant. This group of animals has been difficult to work with as samples are taken from apparently healthy sheep at slaughter and it is not then possible to go back and examine the animal after the result comes through. However, with the Biorad TeSeE detection system, positive signals were being picked up on tests which were negative with other detection systems such as Prionics Check [57,69]. These were investigated carefully and it was found that this group of atypical scrapie cases had abnormal PrP extracted from their brains which was detectable with methods using low levels of proteinase K (such as BioRad TeSeE) and not detectable with the levels of proteinase K which are normally used in the preparation of $PrP^{Sc}$ from classical scrapie cases (such as Prionics Check). Further analysis indicated similarities with Nor98 in the presence of a 12-kDa band on Western blots and in transmission characteristics to transgenic mice [67]. This additional band is shown in Fig. 3b, lane 3, compared with a classical scrapie case (lane 2 and 4). Fig. 3 also shows the differential binding of antibodies to this atypical PrP protein—6H4 does not bind (Fig. 3a, lane 3), whereas P4 does (Fig. 3b, lane 3). The higher molecular weight bands in atypical cases are highly variable in appearance—this is a single example to illustrate the distinct 12 kDa band (Hunter and Chong, unpublished). PrP genetics in these sheep were again surprising with even the previously thought highly resistant ARR/ARR genotype being reported as showing such

Table 6
Criteria for the categorisation of TSEs in small ruminants

| TSE type | Approved rapid screen | Stringent Western blot | Mild PK Western blot | Pathology | Genotype |
|---|---|---|---|---|---|
| Classical scrapie | positive | positive | positive | Grey matter vacuolation IHC labelling of PrP in medulla and DMNV | Susceptible |
| BSE smallin ruminants | positive | positive unglycosylated band lower molecular weight than for classical scrapie controls | positive | Usually grey matter vacuolation. IHC labelling of PrP in medulla and usually also DMNV | Likely to be ARQ/ARQ but not genotype exclusive |
| Atypical scrapie including Nor98 | positive | negative | positive including band at <15 kDa | Vacuolation and IHC negative or low levels in obex, DMNV, medulla. Cerebellum is IHC positive for PrP and has vacuolation | Genotypes include those resistant to classical scrapie often with at least one AHQ or AF141RQ allele |

PK—proteinase K, IHC—immunohistochemistry, DMNV—dorsal motor nucleus of the vagus. Table adapted from [57].

cases [70] (Table 7). Similar cases are now being reported from all over Europe including Great Britain [71].

The European Food Safety Authority (EFSA) has produced a very useful set of definitions of Classical Scrapie, Atypical Scrapie and BSE for small ruminants which is summarised in Table 6 [57,69]. This has allowed individual countries to file reports of case numbers. In the DEFRA report on TSEs in Great Britain for 2005 [72] figures are given for the incidence of both classical and atypical scrapie which are surprising (Table 7). In the abattoir survey in 2005, 11,106 sheep were tested, 12 were found to have classical scrapie and 16 had atypical scrapie signs. These sheep are apparently healthy, destined for the foodchain, so any obviously sick animals would have been removed so perhaps this ratio is not too surprising. However in the fallen stock survey 8271 sheep were tested, 26 had classical scrapie and 6 had atypical scrapie. This gives an overall figure for scrapie in GB in 2005 of 60 cases, of which 22 (37%) were atypical. Why was this not recognized previously? The answer to this question is straightforward: before the rapid testing methods were introduced the standard detection for $PrP^{Sc}$ was to use high levels of proteinase K which would have destroyed the protein signal from the atypical scrapie cases.

With the increased number of cases being reported, the genotypes of animals affected by atypical scrapie have been compared with classical scrapie animals. For 2005 in GB, these results are shown in Table 7. It is clear that this new form of scrapie is appearing in genotypes previously thought to be scrapie resistant and which remain resistant to classical scrapie (Table 8). It is also being found in closed flocks of sheep used for research, such as the DEFRA flock of New Zealand derived animals (announced in a press release in November 2006, www.defra.gov.uk/news/2006/06114a.htm) and in the NPU Cheviot flock which has been closed to new introductions of sheep since 1962 (Foster and Hunter, unpublished). The source of the disease in these latter cases is far from clear. Atypical cases have been reported from all over Europe giving rise to serious questions about the continuing relevance of the National Scrapie Plan and its European counterparts which are selecting

Table 7
Results from targeted (Active) surveillance for TSE in sheep and goats in Great Britain during 2005

| Survey category | Number tested | Number negative | Number classical scrapie | Number atypical scrapie |
|---|---|---|---|---|
| Sheep abattoir survey [a] | 11,106 | 11,078 | 12 | 16 |
| Sheep fallen stock survey [b] | 8271 | 8239 | 26 | 6 |
| Goat abattoir survey [c] | 1282 | 1282 | 0 | 0 |
| Goat fallen stock survey [d] | 1329 | 1325 | 4 | 0 |
| Total sheep | 19,377 | 19,317 | 38 | 22 |
| Total goats | 2611 | 2607 | 4 | 0 |

Data taken from reference [87]. Crown copyright 2006.
[a] Sheep abattoir survey: random selection of 10,000 sheep aged over 18 months and slaughtered for human consumption.
[b] Sheep fallen stock survey: random selection of 10,000 fallen sheep aged over 18 months and notified voluntarily.
[c] Goat abattoir survey: all goats aged over 18 months and slaughtered for human consumption.
[d] Goat fallen stock survey: random selection of 1000 fallen goats aged over 18 months and notified voluntarily.

Table 8
Genotypes of classical and atypical scrapie cases detected through active surveillance between 2002 and 2005 in Great Britain

| Genotype | NSP type | Classical scrapie | Atypical scrapie |
|---|---|---|---|
| Unknown | – | 1 | 0 |
| ARR/ARR | 1 | 0 | 15 |
| ARR/AHQ | 2 | 0 | 29 |
| ARR/ARQ | | 1 | 13 |
| ARR/ARH | | 0 | 1 |
| AHQ/AHQ | 3 | 1 | 13 |
| AHQ/ARH | | 0 | 2 |
| ARQ/ARH | | 1 | 0 |
| ARH/ARH | | 0 | 0 |
| AHQ/ARQ | | 3 | 21 |
| ARQ/ARQ | | 21 | 13 |
| ARR/VRQ | 4 | 31 | 0 |
| AHQ/VRQ | 5 | 0 | 0 |
| ARH/VRQ | | 10 | 0 |
| ARQ/VRQ | | 71 | 1 |
| VRQ/VRQ | | 14 | 0 |
| Total | – | 154 | 108 |

NSP: National Scrapie Plan.
Data from reference [87]. Crown copyright 2006.

apparently for genotypes of sheep which are susceptible to this new and unknown risk of disease. It is not clear whether atypical scrapie is a novel strain of scrapie which has arisen as a result of all the genotype selection being carried out in Europe however this is unlikely in the short timescale. It is more likely that it has been around for much longer and only discovered due to the increased surveillance. In an archive search of sheep samples, a recent atypical scrapie case has been found in a sheep which died in Scotland in 1989 [73].

## 8. Remaining questions

Why do not all sheep of susceptible genotypes succumb to disease—is this because of genotypes defined by codons outside the "three codon" range? Many sheep PrP gene codons are known to vary and more polymorphisms are found every year [74]. Perhaps some of these have an influence on disease progression, such as the increased resistance to experimental BSE linked to codon 168 [52,53]. The possibility is also being investigated that there is DNA variation in regions of the PrP gene which control levels of gene expression and hence protein levels [75]—mouse studies strongly suggest differences in $PrP^{C}$ levels are inversely related to length of incubation period so this is a reasonable suggestion [76,77].

Development of reliable pre-clinical diagnostic tests in the live animal is a major focus of research. Lymphoid tissues are often accessible for biopsy and tonsil [78] and third eyelid [79] have been proposed as sites for detection of $PrP^{SC}$. However the pathogenesis of scrapie in sheep is variable and linked to PrP genotype. Sheep of VRQ/VRQ genotype have widespread distribution of $PrP^{SC}$ in their peripheral lymphoid tissues however sheep of other genotypes, with clinical such as VRQ/ARQ and VRQ/ARR have very little $PrP^{SC}$ in peripheral tissues [29]. Even ARQ/ARQ sheep are variable with some reported as having widespread $PrP^{SC}$ and some which do not [38]. Therefore, using $PrP^{SC}$ as a marker for infection, which most tests do, a positive result gives a good indication of disease being present but a negative result is unreliable. Recently however a very promising new technique has been described which samples lymphoid tissue from the rectal mucosa of sheep and is reported to be genotype independent [80].

There are still remaining questions about the origins of atypical scrapie and how it transmits between sheep, if indeed it does—the old idea of "genetic disease" has arisen again due to the sporadic nature of the occurrence of Nor-98 [66]. Even with classical scrapie, thought to infect animals by the oral route and most likely in the perinatal phase of life, the etiology is uncertain and it may be that many possible routes of infection exist in sheep. Intriguing hints are found occasionally—most recently the association of mastitis in sheep with increased $PrP^{SC}$ deposition in the inflamed tissues has raised the issue of whether milk from animals affected by mastitis can pass infection to their suckling lambs [81]. True maternal (in utero) transmission has been difficult to demonstrate unequivocally, however it is known that placental tissues can harbour and transmit infection by inoculation [82] and recent more detailed studies of $PrP^{SC}$ deposits have found that in scrapie affected ewes, $PrP^{SC}$ is found in placental tissues only if the foetus is of a susceptible genotype [83–85]. This implies some interaction between mother and foetus the molecular and cellular basis of which is again unknown.

Although a great deal of progress has been made in our understanding of the interaction between scrapie and its natural host the sheep, it seems that more research and surveillance, whilst answering some questions, also reveal further unknowns in this intriguing group of diseases. Hopefully there will be much here to interest new young investigators, and indeed the research funders, in future studies.

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